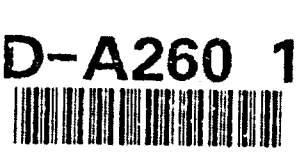

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Plasmodium falciparum: Chymotryptic-like Proteolysis Associated with a 101-kDa Acidic–Basic Repeat Antigen

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NWAGWU, M., HAYNES, J. D., ORLANDI, P. A., AND CHULAY, J. D. 1992. *Plasmodium falciparum:* Chymotryptic-like proteolysis associated with a 101-kDa acidic–basic repeat antigen. *Experimental Parasitology* 75, 399–414. Malaria proteinases appear to function in the release of merozoites from infected erythrocytes and the invasion of merozoites into erythrocytes. Chymostatin, an inhibitor of chymotrypsin-like proteinases, inhibits malaria invasion and also inhibits apparent autoproteolysis of a 101-kDa acidic–basic repeat antigen (p101-ABRA) that is found in the vacuolar space surrounding merozoites in *Plasmodium falciparum*-infected erythrocytes. After purification by a monoclonal antibody (MAb 3D5), p101-ABRA degrades into smaller fragments in the absence of chymostatin. In this study fluorogenic proteinase substrates of the type peptidyl-7-amino-4-trifluoromethyl coumarin with phenylalanine or tyrosine linked to AFC were used to characterize chymotryptic-like activity associated with p101-ABRA. When p101-ABRA from the cell extract of *P. falciparum*-schizont-infected erythrocytes was affinity purified on MAb 3D5 beads, chymotryptic-like activity bound to the beads. Seventy-four percent to 96% of the activity detected using MeOSuc–KLF–AFC, Suc–LLVY–AFC, or SY–AFC at a pH optimum of 7.0 was removed from the extract and 6 to 33% was detected on the washed beads. Attempts to recover active enzyme eluted from the beads were not successful. Enzymes cleaving two other substrates (MeOSuc–AAPM–AFC and F–AFC) did not significantly bind to mAB 3D5 beads. Chymotryptic-like activity was also associated with p101-ABRA in fractions from sequential DEAE–Sephacel chromatography, Sephacryl S-200 chromatography, and non-denaturing polyacrylamide gel electrophoresis.

INDEX DESCRIPTORS AND ABBREVIATIONS: *Plasmodium falciparum:* Malaria; Antigens; Acidic–basic repeat antigen (ABRA); 101-kDa merozoite surface antigen (p101-ABRA); Vaccine candidate antigen; Monoclonal antibody (mAB); Proteinase, protease, proteolysis, or endopeptidase; Aminopeptidase; Purification; Enzyme overlay membranes; 7-Amino-4-trifluoromethyl coumarin (AFC); NT buffer (20 m*M* NaCl, 100 m*M* Tris–HCl, pH 7.0); Peptide-AFC fluorogenic substrates are designated with the standard amino acid single-letter code, e.g., SY–AFC is NH_2Seryl-Tyrosyl-AFC; Ethylenediaminetetraacetic acid (EDTA); Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE); Phenylmethylsulfonylfluoride (PMSF); Polyethyleneglycol (PEG); Tris(hydroxymethyl)aminomethane (Tris); benzyloxycarbonyl (Z).

INTRODUCTION

A vaccine is needed to protect against malaria, a major disease that is responsible for over a million deaths annually in several tropical regions. *Plasmodium falciparum*, the principal causative agent, expresses many antigens at its surface which are accessible to circulating antibodies and immunocompetent cells and therefore are candi-

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dates for the development of such a vaccine. In addition to accessibility, it is desirable to target antigens that embody functions critical for parasite survival.

Stage-specific proteinases of *P. falciparum* are needed in biological functions of the parasite stage in which they appear. In this paper we use the term proteinase to describe enzymes acting on foreign proteins or synthetic peptidyl substrates even when the natural protein substrate(s) is not known.

The 101-kDa acidic basic repeat antigen (p101-ABRA) is a soluble antigen located at the merozoite surface, within the parasitophorous vacuole of *P. falciparum* (Chulay *et al.* 1987). Its primary structure (Weber *et al.* 1988) was shown to be identical to the acidic–basic repeat antigen, ABRA, containing repeats of glutamic acid(E) and lysine(K) (primarily KE and KEE), reported by Stahl *et al.* (1986). After its purification by using a mAB 3D5 affinity column, p101-ABRA underwent proteolysis, possibly autoproteolysis, that could be prevented by chymostatin (Weber *et al.* 1988). Preliminary experiments also showed that p101-ABRA was one of several *P. falciparum* antigens that bound to a chymostatin–polyacrylamide affinity gel, suggesting that it might behave as a chymotryptic-like proteinase (Haynes and Chulay, unpublished).

Chymotryptic-like parasite proteinase(s) have been implicated in both the release of merozoites from infected erythrocytes and the invasion of merozoites into erythrocytes (Banyal *et al.* 1981; Tharavanij *et al.* 1983; Dejkriengkraikhul *et al.* 1983; Hadley *et al.* 1983; Dluzewski *et al.* 1986; Lyon and Haynes 1986).

The purpose of this investigation was to determine whether p101-ABRA had proteinase activity. Chymotryptic-like proteinase activity was always associated with p101-ABRA after its partial purification by a variety of methods.

Materials and Methods

All fluorogenic substrates (peptides coupled to 7-amino-4-trifluoromethyl coumarin (AFC) and the enzyme overlay membrane impregnated with SY-AFC) were products of Enzyme Systems Products (Dublin, CA). The enzyme inhibitors, antipain, chymostatin, leupeptin, and phenylmethylsulfonylfluoride (PMSF) were obtained from Sigma Chemical Co. (St. Louis, MO). The disodium salt of ethylenediaminetetraacetic acid (EDTA), polyethylene glycol 6000 (PEG), and $CaCl_2$, were products of Fisher Scientific (Pittsburgh, PA). Affigel-10, tris(hydroxymethyl)aminomethane (Tris) and all the materials and chemicals for electrophoresis were obtained from BioRad Laboratories (Richmond, CA). DEAE–Sephacel and Sephacryl S-200 were products of Pharmacia LKB Biotechnology (Uppsala, Sweden). Immunochemical reagents were obtained from Promega (Madison, WI). Centricon 10 microconcentrators were purchased from Amicon (Danvers, MA). DE81 DEAE ion exchange paper was purchased from Whatman. All other chemicals were of analytical grade.

Parasites. Cloned Camp strain of *P. falciparum* parasites (Malaysia) were cultured and synchronized by double sorbitol treatment as described by Vernes *et al.* (1984). Parasites were harvested by centrifugation of schizont-infected erythrocytes in Percoll–sorbitol gradients (Aley *et al.* 1984). By this method parasitized erythrocytes, 98% enriched in schizonts, were obtained.

Cell extract. A cell extract of *P. falciparum* was prepared by resuspending schizont-infected erythrocytes in NET buffer (150 m*M* NaCl, 1 m*M* EDTA, 50 m*M* Tris–HCl, pH 8.0), followed by two cycles of freezing the suspension at −80° for 1 to 2 hr and thawing at 37°C for 5 min. The samples were centrifuged at 13,000*g* for 2 min at 25°C. The supernatant was recovered and served as cell extract and source of p101-ABRA. A control extract of uninfected erythrocytes was similarly prepared.

Proteinase assays. An appropriate enzyme source was incubated with 10 to 100 μ*M* of a fluorogenic substrate in buffer (20 to 400 m*M* NaCl and 100 m*M* Tris–HCl, pH 7.0) for 15 to 18 hr at 25°C or 37°C in 0.5 ml. More buffer was added to bring the volume of the mixture to 1.0 ml and the concentration of liberated AFC was determined spectrofluorometrically at excitation and emission wavelengths of 368 and 494 nm, respectively. The readings of blank control samples without an enzyme source were included in each assay and were subtracted from those of experimental samples. Known concentrations of free AFC were used to standardize the assay.

Immunoprecipitation. mAB 3D5, which specifically recognizes p101-ABRA, was coupled to Affigel-10

beads, blocked with ethanolamine (Chulay *et al.*, 1987), and stored in NET buffer containing 0.2% sodium azide at 4°C. Blank Affigel for use in control experiments was similarly treated with all the reagents used in the coupling reaction, but without any antibody present. Monoclonal antibody mAB 3D5-Affigel was washed three times with NETT buffer (150 m*M* NaCl, 1 m*M* EDTA, 50 m*M* Tris–HCl, pH 8.0, 0.5% Triton X-100). Cell extracts or other purified chromatographic fractions suspected to contain p101-ABRA were reacted with 40 to 80 μl of mAB 3D5-Affigel beads or, in control experiments, with an equal volume of blank Affigel. Tubes were mixed on a rotator at 25°C for 1 hr, centrifuged at 13,000*g* for 2 min, and the supernatant was discarded. The beads were washed four times: twice with NETT buffer, once with 350 m*M* NaCl, 1 m*M* EDTA, 50 m*M* Tris–HCl, pH 8.0, 0.5% Triton X-100, and finally with NET buffer. The beads with bound material were mixed in buffer (20 m*M* NaCl, 100 m*M* Tris–HCl, pH 7.0) with substrates at 25°C for 15 hr. More buffer was added to a final volume of 1.0 ml, the tubes were centrifuged, and liberated AFC in the supernatant was measured spectrofluorometrically.

Chromatography. p101-ABRA was precipitated from the cell extract by adding PEG. A stock solution of 50% PEG in NT buffer (20 m*M* NaCl, 100 m*M* Tris–HCl, pH 7.0) was added to a final concentration of 30% PEG and mixed for 1 hr at 25°C. The proteinase-enriched precipitate was pelleted by centrifugation, dissolved in NT buffer, and layered on a 10-ml column of DEAE–Sephacel equilibrated with NT buffer. After 20 ml of NT buffer, a 120-ml linear gradient of 20 m*M* to 1.0 *M* NaCl in 100 m*M* Tris–HCl, pH 7.0, was applied. Fractions (about 2 ml each) were collected and the proteinase activity of an aliquot, 200 μl, was determined. Fractions of peak activity were pooled and concentrated with Amicon Centricon 10.

The concentrated sample obtained after DEAE–Sephacel chromatography was layered on a column of Sephacryl S-200 equilibrated with buffer (150 m*M* NaCl, 100 m*M* Tris–HCl, pH 7.0) and fractions (2 ml) were collected at a flow rate of 30 ml/hr at 4°C. The proteinase activity of an aliquot of each fraction was determined. For some experiments, fractions of peak activity were pooled and concentrated with Amicon Centricon 10. For the determination of the M_r values, the column was calibrated with thyroglobulin, aldolase, bovine serum albumin, and chymotrypsinogen of respective M_r values, 669, 158, 67, and 25 kDa.

Analytical SDS–PAGE and Western blotting. Discontinuous SDS–PAGE was performed as described by Laemlii (1970) on 1.5 mm 7.5% polyacrylamide. The samples were heated with equal parts of 2 × SDS sample buffer, loaded, and electrophoresed at 8 mA per gel, at 15°C for 15 hr. For Western blotting, antigens were electroblotted onto nitrocellulose filters, blocked with TBS–0.3% Tween 20 for 90 min, probed with monoclonal antibody mAB 3D5 (Lyon *et al.* 1987; Chulay *et al.* 1987), and then developed using an alkaline phosphatase-conjugated anti-mouse immunoglobulin second antibody.

Nondenaturing gel electrophoresis. Cell extract or partially purified p101-ABRA obtained after DEAE–Sephacel chromatography was fractionated by electrophoresis on polyacrylamide gels in the system of Laemlii (1970) as above, except that SDS was omitted and the temperature was maintained at 4°C. The gels were blotted at 4°C for 2 hr 30 min onto DEAE paper (McLellan and Ramshaw 1981) in the blotting system of Towbin *et al.* (1979) but in the absence of methanol. The DEAE paper was then cut into 0.5-cm horizontal strips, transferred into tubes containing 2.0 ml of elution buffer (400 m*M* NaCl, 100 m*M* Tris–HCl, pH 7.0), vigorously stirred, incubated on ice for 1 hr, and centrifuged at 3000*g* for 30 min. The supernatant was examined by proteinase assays, SDS–PAGE, western blotting, and silver staining (BioRad).

Proteinase activity in nondenaturing gels was also observed by the enzyme overlay membrane method of Smith (1984) and Smith *et al.* (1988). The procedure provided by Enzyme Systems Products (1989) was followed. The cell extract of schizont-infected erythrocytes or chromatographic fractions of the extract were analyzed on nondenaturing, reducing polyacrylamide gels 0.75 mm thick, otherwise as described above. Immediately after electrophoresis, an enzyme overlay membrane impregnated with SY–AFC was rapidly dipped into distilled water and carefully laid over the gel and incubated at 37°C in a humidified chamber. The green fluorescence appearing from the enzymatic reaction was monitored, and when maximum fluorescence was reached (usually within 72 min), the membrane was peeled off and photographed under ultraviolet light.

RESULTS

Proteinase activities in freeze-thawed cell extract from schizont-infected erythrocytes were examined using several fluorogenic peptide substrates. Proteinases in the cell extract were active against peptidyl-AFC substrates with phenylalanine (F), tyrosine (Y), methionine (M), arginine (R), proline (P), or leucine (L) at the carboxyl terminus of the peptide just before the AFC group (Table I).

Because it was suspected that p101-ABRA was a chymotrypsin-like proteinase, and chymotrypsin primarily catalyzes the hydrolysis of amide bonds of proteins and

TABLE I
Proteinase and Aminopeptidase Activities of Cell Extracts Prepared from *P. falciparum* Schizont-Infected Erythrocytes or from Uninfected Erythrocytes

Substrate	Specific activity (nM AFC/10⁶ schizonts)	(nM AFC/10⁶ erythrocytes)
Bz-VKKR-AFC	800	—
F-AFC	500	—
SY-AFC	400	24
MeOSuc-KLF-AFC	250	—
MeOSuc-AAPM-AFC	125	—
Suc-LLVY-AFC	25	6
Glut-GGF-AFC	24	—
GP-AFC	24	—
Z-RGFFL-AFC	18	—
Glut-Y-AFC	5	—
MeOSuc-AAPV-AFC	0	—
MeOSuc-FPF-AFC	0	—

Note. The proteinase activity of the cell extract was determined by measuring the amount of AFC cleaved from the peptide substrates in the standard reaction mixture described under Materials and Methods at a substrate concentration of 100 to 200 μ*M*. The results represent averages of at least two experiments, each of which was carried out in duplicate. —, not done.

peptides adjacent to the carboxyl group of the aromatic L-amino acid residues of phenylalanine and tyrosine (and to a lesser extent tryptophan), attention was focused on the enzymatic activity against the peptide substrates MeOSuc-KLF-AFC, SY-AFC, and Suc-LLVY-AFC.

mAB 3D5 coupled to Affigel beads was used to affinity purify p101-ABRA from cell extracts. The beads were then washed and assayed for chymotryptic-like proteinase activity with MeOSuc-KLF-AFC. Chymotryptic-like proteinase(s) of cell extract obtained from 1–2 × 10^8 schizont-infected erythrocytes maximally bound to 80 μl of mAB 3D5 affinity gel (Fig. 1a) and the optimal pH of the reaction was 7.0 (Fig. 1b). Varying NaCl concentrations had little effect (data not shown). These data support the hypothesis that p101-ABRA is a chymotryptic-like proteinase, and these optimal conditions were used in later experiments.

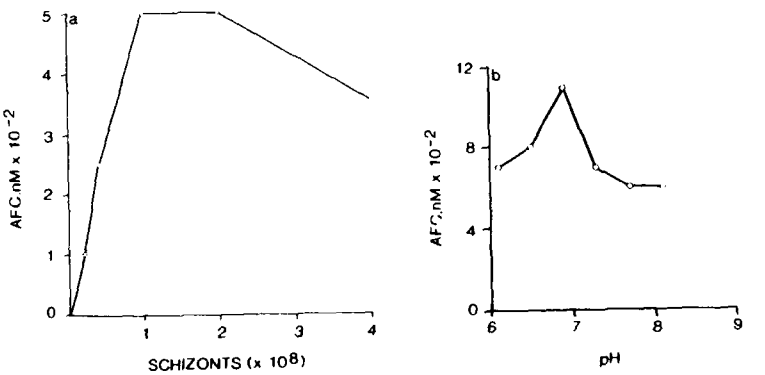

FIG. 1. Proteinase activity is reported as the amount of AFC released from MeoSuc-KLF-AFC in the standard enzymatic reaction for 15 hr at 25°C. (a) Binding of chymotryptic-like activity to mAB 3D5 beads and the amount of cell extract from schizont-infected erythrocytes required to saturate binding. The cell extract obtained from different quantities of schizont-infected erythrocytes was incubated with 80 μl of mAB 3D5–Affigel for 30 min at 25°C. Unadsorbed material was washed off as described under Materials and Methods and the mAB 3D5–Affigel for 30 min at 25°C. Unadsorbed material was washed off as described under Materials and Methods and the mAB 3D5–Affigel with bound antigens was incubated with substrate. (b) Dependence on pH of the proteinase activity of the antigen(s) bound to mAB 3D5. The cell extract obtained from 2 × 10^8 schizont-infected erythrocytes was incubated with mAB 3D5–Affigel for 30 min at 25°C. At the end of the reaction the beads were washed and incubated with substrate in the presence of buffers of different pH values.

In preliminary experiments, the substrates MeOSuc–KLF–AFC, SY–AFC and Suc–LLVY–AFC detected similar chymotryptic-like proteinase activity from *P. falciparum* extracts: in addition to their being acted upon by the enzymes(s) bound by mAB 3D5, the chromatographic profiles of the enzyme(s) active against them were similar in parallel experiments (data not shown).

The ability of mAB 3D5 to bind chymotryptic-like activity (Fig. 1A) was further investigated using fractions from Sephacryl S-200 chromatography of schizont-infected erythrocyte cell extract (Table II). Aliquots of peak tubes with different average rela-

TABLE II
Chymotryptic-like Proteinase of *P. falciparum*: Binding to mAB 3D5 Beads

Fraction	Relative specific activity[a]	Before adsorbed to mAB[b]	After adsorbed to mAB 3D5 beads: % Removed from supernatant	After adsorbed to mAB 3D5 beads: % Recovered on washed beads
		Chymotryptic-like activity (Suc–LLVY–AFC)		
≥200 kDa	5.04	287	75	33
~90 kDa	1.50	1,134	74	5.9
~38 kDa	0.05	287	ND	ND
~12 kDa	0.81	675	96	5.2
		Aminopeptidase activity (F–AFC substrate)		
≥200 kDa	0.49	172	0	0
~90 kDa	3.10	15,026	0	0
~38 kDa	0.23	8,137	ND	ND
~12 kDa	0.31	1,536	0	0

Note. ND, not determined.

[a] The specific activity of each fraction from a Sephacryl S-200 column was divided by the specific activity of the cell extract before it was applied to the column. Specific activity = (pmol AFC released)/mg protein. One milligram of protein was estimated as 1 ml with an OD_{280nm} of 1. Protein eluted as a broad peak centered at about 38 kDa; the chymotryptic-like activity and the aminopeptidase both peaked in the ~90-kDa fraction. Note, however, that the activity in the ≥200-kDa fraction eluted in the void volume.

[b] Enzymatic activity is picomoles of AFC liberated during assay. Cell extract (500 μl) obtained from 5×10^8 schizont-infected erythrocytes was fractionated on a column (1.8 × 30 cm) of Sephacryl S-200. Fractions (2.4 ml) were eluted with buffer, 0.75 *M* NaCl, 100 m*M* sodium phosphate buffer, pH 6.5. Aliquots of the starting extract (10 μl) or fractions (250 μl) from tubes were analyzed for enzymatic activities as described under Materials and Methods. Of the 133,455 units of LLVY–AFC activity (picomoles AFC released during 18 hr at 25°C) applied to the S-200 column, 68,057 (51%) were recovered. Of the LLVY–AFC activity recovered from the column, 8.4% was in the void volume peak (≥200 kDa), 34.4% was in a peak centered at about 90 kDa, and 35.8% was in a peak centered at about 12 kDa, with the rest trailing out several tubes beyond the lower resolution of S-200. Of the 861,000 units of F–AFC activity (picomoles AFC released during 18 hr at 25°C) applied to the column, 523,884 (61%) were recovered, most of it in a broad peak centered at about 90 kDa, with only 0.3% ≥200 kDa and 5.1% ~12 kDa. Activities in single tubes at the peaks of proteinase activity were further analyzed for binding to the anti-p101-ABRA mAB, 3D5. Aliquots (250 μl) of each fraction were incubated with 200 μl of mAB 3D5–Affigel beads for 1 hr at 25°C. Beads were washed three times with column buffer, and the activity of bound enzyme was determined as described under Materials and Methods. Each fraction was also reacted with control Affigel beads which were similarly processed. Percentage specifically removed by mAB 3D5 was calculated by subtracting activity remaining in supernatant after adsorption with 3D5 beads from the activity remaining after adsorption with control beads, and the result was divided by activity remaining after adsorption with control beads—this calculation corrects for dilutional losses of activity in the beads. To determine the percentage bound, the small amount of enzyme activity retained on control Affigel beads was subtracted from the activity on the mAB 3D5 beads, and the result was then divided by the starting activity.

tive molecular sizes were adsorbed to mAB 3D5 beads. Consistent with earlier results, 74 to 96% of the chymotryptic-like activity against Suc–LLVY–AFC was removed by adsorption to mAB 3D5 beads, and 5 to 33% was detected bound to washed beads (Table II). By contrast, as a control for specificity of binding, no aminopeptidase activity against F–AFC was removed from supernatants or could be detected binding to mAB 3D5 beads (Table II).

Even though about 50% of the chymotryptic-like activity was lost during this purification on Sephacryl S-200, the relative specific activity per milligram protein increased up to fivefold compared with the starting cell extract. The relative specific activity of the >200-kDa fraction (5) was 100 times that of the 38-kDa fraction (0.05) (Table II).

p101-ABRA, apparently as complexes with itself and/or other molecules, was detected in the proteinase peak of $M_r \geq 200$ kDa (by immunoprecipitation and immunoblotting with mAB 3D5, data not shown).

In additional experiments with unfractionated schizont extract, 6 to 23% of the activities measured with the two other chymotrypsin substrates, MeOSuc–KLF–AFC and SY–AFC, were detected bound to

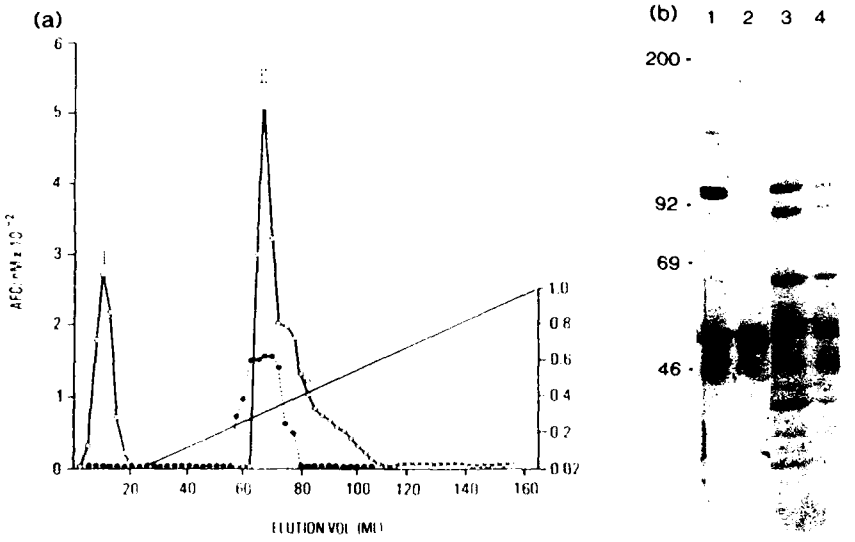

FIG. 2. DEAE–Sephacel chromatography of a cell extract of schizont-infected erythrocytes and uninfected erythrocytes. (a) The cell extract obtained from 8×10^8 schizont-infected erythrocytes or from the same number of uninfected erythrocytes was layered, in separate experiments, on DEAE–Sephacel columns of 8 ml volume equilibrated with NT buffer and eluted as described under Materials and Methods. Proteinase activity was determined with SY–AFC as substrate and is given per 200-μl aliquot of a 2-ml fraction. Similar results were obtained with Suc–LLVY–AFC as substrate. All other conditions, were as given under Materials and Methods. ○—○, schizont-infected erythrocytes; ● · · · ●, uninfected erythrocytes. (b) Western immunoblot analysis of chymotrypsin-like proteinase(s) of peaks I and II of a. Peaks I and II of a were separately pooled and concentrated with Centricon 10. After SDS–PAGE the proteins were blotted, blocked, incubated with mAB 3D5 (1 μg/ml) for 15 hr at 25°C, washed, and developed using alkaline phosphatase-conjugated anti-mouse antibody. Lane 1, cell extract from 2.5×10^6 schizont-infected erythrocytes; lane 2, cell extract from 2.5×10^6 uninfected erythrocytes; lanes 3 and 4, peaks II and I, respectively, from DEAE chromatography (a) of chymotrypsin-like proteinase(s) equivalent to a starting material obtained from 1.3×10^7 schizont-infected erythrocytes.

mAB 3D5 beads after washing. In another control demonstrating specificity of binding, less than 0.1% of the proteinase activity against MeOSuc–AAPM–AFC bound to mAB 3D5 (data not shown).

Cell extract was chromatographed on DEAE–Sephacel and fractions were analyzed for chymotryptic activity. Two peaks of activity were observed using the substrates SY–AFC (Fig. 2a, peaks I and II) or Suc–LLVY–AFC (not shown). The presence of p101-ABRA in both peaks, and also some smaller molecular weight products and a few faint cross-reacting bands of higher molecular weight, was demonstrated by immunoblotting with mAB 3D5 (Fig. 2b, lanes 3 and 4). In subsequent experiments peak II was eluted with buffer, 400 m*M* NaCl, 100 m*M* Tris–HCl, pH 7.0, after collecting the unadsorbed peak I.

Each peak was further purified by gel filtration on a Sephacryl S-200 column (Fig. 3). Activity in peak I eluted from S-200 columns around 90 to 110 kDa; activity in peak II consistently eluted as a peak of $M_r \geq 200$ kDa.

The effects of protease inhibitors were determined on the chymotryptic-like activity from DEAE peak I eluting at about 90 kDa from Sephacryl S-200 (Table III). The chymotrypsin-like proteinase was 88% inhibited by chymostatin (1 m*M*), a classic inhibitor of chymotrypsin. Leupeptin or antipain (1 m*M*), inhibitors of some serine and cysteine proteinases, not including chymotrypsin, inhibited the proteinase by 53 to 67%. PMSF, 1 mM, another good inhibitor of many serine proteinases including chymotrypsin, was only slightly inhibitory. Calcium chloride and EDTA had no effect.

Extracts of uninfected erythrocytes did not contain any chymotryptic-like activity equivalent to peak I in extracts of schizont-infected erythrocytes (Figs. 2a and 3). Immunoblots of extracts of uninfected erythrocytes with mAB 3D5 revealed no bands in the 100-kDa region or above 60 kDa (Fig. 2b, lane 2). There was a small amount of chymotryptic-like activity in extracts of normal uninfected erythrocytes (Table I, last column) that eluted near the peak II parasite proteinase from DEAE–Sephacel (Fig. 2a, erythrocyte proteinase, dotted line) and from Sephacryl S-200 as a molecule of $M_r \geq 200$ kDa (Fig. 3, triangles).

In order to avoid contamination with erythrocyte enzyme, we concentrated next on the M_r ~90 kDa fractions from parasitized erythrocytes, which were shown to contain only parasite enzyme. Individual fractions of the ~90-kDa chymotrypsin-like proteinase peak, instead of the pooled peak, were analyzed to determine the extent of purification. In the most highly pu-

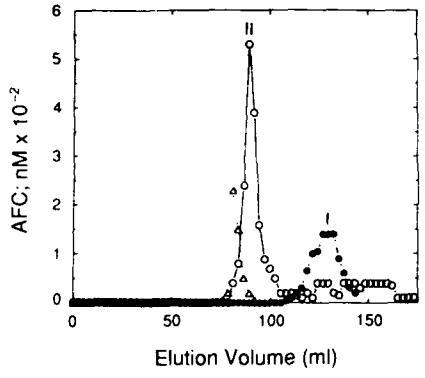

FIG. 3. Gel filtration of peaks I and II of chymotrypsin-like proteinase of schizont-infected erythrocytes following DEAE–Sephacel chromatography. Following the chromatography of the cell extract of schizont-infected erythrocytes (8×10^8 parasitized erythrocytes) on DEAE–Sephacel, peaks I and II of chymotrypsin-like proteinase(s) of Fig. 2a were separately pooled, concentrated, and layered on a column of Sephacryl S-200 (2.5 × 45 cm). The DEAE–Sephacel peak of chymotrypsin-like proteinase of uninfected erythrocytes (8×10^8) was similarly analyzed. Fractions, 2.7 ml, were eluted with buffer, 400 m*M* NaCl, 100 m*M* Tris–HCl, pH 7.0. The activity, given per 200-μl aliquot, was determined with SY–AFC as substrate. ●—●, DEAE–Sephacel peak I of schizont-infected erythrocytes (contains chymotrypsin-like proteinase(s) of M_r ~90 kDa); ○—○, DEAE–Sephacel peak II of schizont-infected erythrocytes (contains $M_r \geq 200$-kDa chymotrypsin-like proteinase(s)). △ · · · △, DEAE–Sephacel protease peak of uninfected erythrocytes.

TABLE III
The Effects of Inhibitors on the Activity of the ~90-kDa Peak of Chymotrypsin-like Proteinase

Inhibitor	Final concentration	Substrate	Percentage inhibition
$CaCl_2$	1 m*M*	Suc-LLVY-AFC	0
		Suc-LLVY-AFC	0
EDTA	1 m*M*	Suc-LLVY-AFC	0
PMSF	1 m*M*	Suc-LLVY-AFC	18
Chymostatin	50 μ*M*	Suc-LLVY-AFC	0
	175 μ*M*	Suc-LLVY-AFC	50
	175 μ*M*	MeOSuc-KLF-AFC	36
	175 μ*M*	HCl-SY-AFC	45
	1 m*M*	Suc-LLVY-AFC	88
Leupeptin	1 m*M*	Suc-LLVY-AFC	53
Antipain	1 m*M*	Suc-LLVY-AFC	67

Note. The cell extract from 3×10^8 schizont-infected erythrocytes was precipitated with 30% polyethylene glycol and redissolved in NT buffer (20 m*M* NaCl, 100 m*M* Tris–HCl, pH 7.0). The material that did not bind to a DEAE-Sephacel column equilibrated with NT buffer (peak I) was concentrated and further purified by gel filtration on Sephacryl S-200. The ~90-kDa peak of chymotrypsin-like proteinase was pooled and assayed in an incubation mixture containing 50 μl of the ~90-kDa peak, 5 μ*M* of peptide–AFC, with or without inhibitor (concentration as given), and NT buffer to make 1 ml. The tubes were incubated at 37°C for 18 hr after which the amount of liberated AFC was determined spectrofluorometrically.

rified fraction of M_r ~110 kDa from the ascending limb of the ~90-kDa proteinase peak, silver staining revealed a polypeptide band of 101 kDa (Fig. 4A) and immunoblotting with mAB 3D5 revealed p101-ABRA (Fig. 4B) with few contaminating bands other than what may be autoproteolytic products of p101-ABRA itself.

Other separation schemes were used in the attempt to establish correspondence between the chymotrypsin-like proteinase(s) and p101-ABRA of schizont-infected erythrocytes. For example, the chymotrypsin-like proteinase(s) was separated on polyacrylamide gels under nondenaturing conditions, blotted onto DEAE paper, eluted from the paper, and assayed for proteinase activity. Similar results were found for unpurified extract (Fig. 5A) and for peak I (~90 kDa) enzyme activity from DEAE–Sephacel (not shown): the chymotrypsin-like proteinase(s) eluted from the paper as a peak between 2 and 4 cm from the top. Upon reelectrophoresis and assay, each 0.5-cm fraction from 1.5 to 3.5 cm maintained its activity and eluted in its original position (data not shown). In contrast, the p101-ABRA in peak II associated with >200-kDa complexes did not appear to enter the nondenaturing gel, and no activity was detected in nondenaturing electrophoresis gels of peak II.

The activity of the chymotrypsin-like proteinase separated on nondenaturing polyacrylamide gels was also determined *in situ* by overlaying membranes impregnated with SY–AFC on the gel and visualizing the fluorescence of the AFC released in the enzymatic reaction. The region of fluorescence in the membranes (Fig. 5B, lane 1) correspond with the same region of the gel where the chymotrypsin-like proteinase fractions containing p101-ABRA banded, between 2 and 4 cm from the top of the gel (Figs. 5A and 5B). By contrast there was no fluorescence in the lanes containing extracts of uninfected erythrocytes (Fig. 5B, lane 3), showing that the observed fluorescence was due to the activity of parasite chymotryptic-like enzyme.

DEAE peak I proteinase activity was separated by nondenaturing gel electropho-

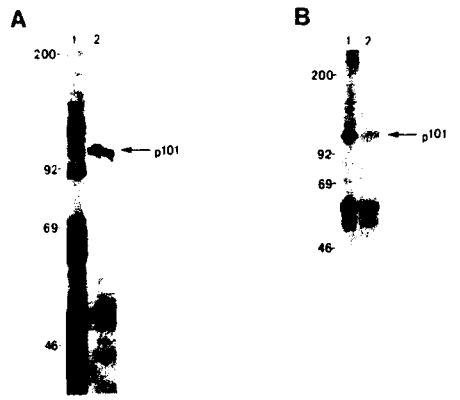

FIG. 4. Silver stain and immunoblot of SDS–PAGE of Sephacryl S-200-purified chymotrypsin-like proteinase(s) of M_r 110 kDa. A cell extract of schizont-infected erythrocytes (1×10^9 parasitized cells) was prepared in NET buffer containing chymostatin and leupeptin at a concentration of 25 μg/ml each. Proteins were precipitated with polyethylene glycol and the pellet was dissolved in 20 m*M* NaCl, 100 m*M* Tris–HCl, pH 7.0, containing chymostatin and leupeptin, 25 μg/ml, each, and chromatographed on DEAE–Sephacel. Peak I was collected, concentrated, and purified on Sephacryl S-200 as described under Materials and Methods. Fractions were collected and analyzed for chymotryptic-like activity, and the active fractions individually concentrated and analyzed in duplicate by SDS–PAGE. (A) Silver stain. Lane 1, polyethylene glycol-precipitated cell extract prepared from 2×10^6 schizont-infected erythrocytes; lane 2, fraction of the chymotrypsin-like proteinase(s) of M_r 110 kDa. (B) Western blot analysis with mAB 3D5, lanes the same as in A.

resis and fractions corresponding to enzyme activity were blotted onto DEAE paper (as in Fig. 5) and then analyzed by SDS–PAGE (Fig. 6). Those gel fractions containing chymotryptic-like activity (for example, that from 2 to 2.5 cm) each contained p101-ABRA as determined by silver staining (Fig. 6A, lane 2) and immunoblotting with mAB 3D5 (Fig. 6B, lane 2), and these fractions contain fewer proteins than the unfractionated cell extract (compare with lane 1 of Fig. 6A).

DISCUSSION

Fluorogenic substrates were used to detect proteinases (using peptidyl-AFC) and an aminopeptidase (F-AFC) in the supernatant of freeze–thaw extract of schizont-infected erythrocytes (Table I). Freezing and thawing released soluble proteins such as p101-ABRA, without the additional proteins that would have been released by detergent lysis. Because the existence and biological importance of malaria parasite enzymes with chymotryptic-like activity had been postulated based upon the biological effects of inhibitors of chymotrypsin (Dejkriengkraikhul and Wilairat 1983; Hadley, *et al.* 1983; Dluzewski *et al.* 1986; Lyon and Haynes 1986; and Deplace *et al.* 1988), we concentrated on those substrates that allowed the assay of chymotryptic-like activity.

Because preliminary work had indicated that p101-ABRA might be a chymotryptic-like proteinase, we used a monoclonal antibody (mAB 3D5) against p101-ABRA (Chulay *et al.* 1987). When coupled to Affigel beads, mAB 3D5 consistently removed most of the chymotryptic-like proteolytic activity, but *not* other enzymatic activities, from parasite extracts (Table II and additional experiments). Unfortunately, we were unable to efficiently elute enzyme that remained active from mAB 3D5 beads. Loss of activity seemed to occur during or shortly after elution with any of several buffers and pHs (data not shown).

Losses of total chymotryptic-like activity were also noted during other purification methods, but enough chymotryptic-like activity remained to correlate it with the presence of p101-ABRA. For example, after chromatography on DEAE–Sephacel (Fig. 2) and then Sephacryl S-200 (Fig. 3), a fraction containing proteins of about 110 kDa was obtained from the 90-kDa peak containing chymotryptic-like activity (ascending limb of peak I of Fig. 3). SDS–PAGE of this fraction followed by silver staining (Fig. 4A, lane 2) showed that p101-ABRA was purified compared to starting material (Fig. 4A, lane 1); its identity was confirmed

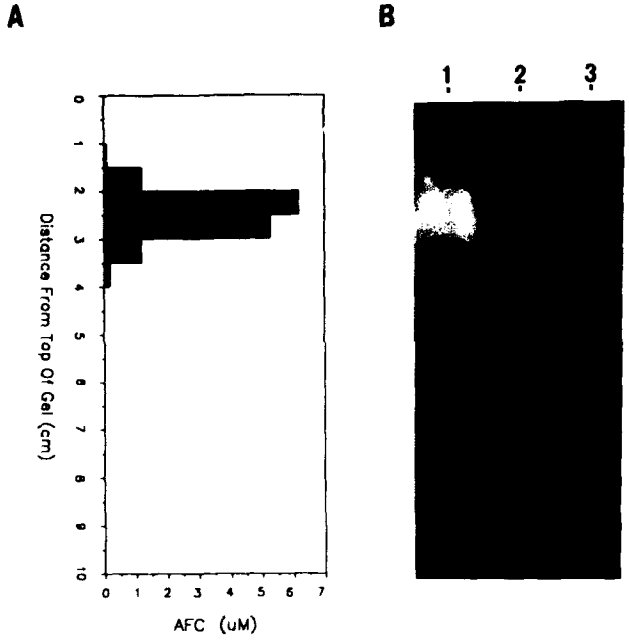

FIG. 5. Electrophoresis of active enzyme on nondenaturing, reducing preparative polyacrylamide gels. (A) A cell extract (1 ml) from 1×10^9 schizont-infected erythrocytes in NET buffer (pH 8) containing chymostatin and leupeptin, 25 μg/ml each, was concentrated to 160 μl, mixed with an equal volume of 2× electrophoresis sample buffer without SDS, and run on a 7.5% polyacrylamide gel with a 3.5% stacking gel (without SDS). The gel was blotted onto DEAE paper which was then cut into 0.5-cm horizontal strips, beginning from the top to the bottom, and the proteins were eluted. An aliquot, 200 μl, of the eluted proteins was transferred to a tube and chymotrypsin-like proteinase activity was determined with SY–AFC as substrate. (B) Enzyme-overlay membranes impregnated with SY–AFC were used to visualize chymotrypsin-like proteinase activity in nondenaturing gels. Cell extracts of uninfected erythrocytes and schizont-infected erythrocytes were prepared in NET buffer (pH 7) and aliquots equivalent to extracts of 4×10^7 schizont-infected or uninfected erythrocytes were loaded onto a 7.5% nondenaturing, reducing polyacrylamide gel, 0.75 cm thick. Visualization of chymotryptic-like proteinase(s) were performed as described under Materials and Methods. Lane 1, schizont-infected erythrocytes; lane 2, blank (sample buffer); lane 3, uninfected erythrocytes.

by Western blotting with mAB 3D5 (Fig. 4B, lane 2). Some of the lower molecular weight bands are probably cleavage products of p101-ABRA, as noted previously (Chulay *et al.* 1987, and unpublished). A Western blot of uninfected erythrocytes detected only small amounts of a few low molecular weight molecules (Fig. 2B, lane 2).

It seems likely that inherent proteolytic activity of p101-ABRA contributed to molecular hetereogeneity by creating fragments of itself (and other proteins) during purification. Losses of activity during purification and storage overnight at 4°C were found (data not shown), although, perhaps similar to some proteinases that retain some activity after processing to smaller sizes, some activity appeared in lower molecular weight fractions (see Table II). Performing the purifications at 4°C and including reversible proteinase inhibitors in the early stages of purification did not completely solve these problems.

Under nondenaturing conditions one en-

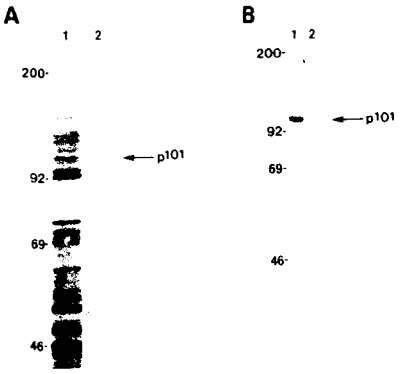

FIG. 6. SDS–PAGE analysis of enzymatically active fractions sequentially purified on DEAE Sephacel (the M_r ~90 kDa peak I) and on nondenaturing, reducing polyacrylamide gel. A cell extract was prepared from 9.5×10^8 schizont-infected erythrocytes in the presence of chymostatin and leupeptin, 25 μg/ml each, and layered on a column of DEAE–Sephacel, 10 ml volume, equilibrated with 20 m*M* NaCl, 100 m*M* Tris–HCl, pH 7.0, containing chymostatin and leupeptin, 25 μg/ml, each. The unadsorbed eluate containing chymotrypsin-like proteinase(s) of M_r ~90 kDa was collected, concentrated, and run on a nondenaturing, reducing, preparative polyacrylamide gel. After electrophoresis the proteins were blotted onto DEAE paper, eluted, and aliquots tested for chymotryptic-like activity using SY–AFC. The fractions of peak proteinase activity were individually concentrated to a final volume of 50 μl. (A) Silver stain. An aliquot, 20 μl, was analyzed by SDS–PAGE. Lane 1, cell extract from 1×10^6 schizont-infected erythrocytes; lane 2, partially purified chymotrypsin-like proteinase(s) peak I fraction migrating in the nondenaturing gel in a position between 2 and 2.5 cm from the top. (B) Western immunoblot with mAB 3D5. Lanes the same as in A.

zymatically active fraction from DEAD chromatography ran as a large, >200 kDa, molecular size on Sephacryl S-200 gel exclusion chromatography (peak II of Figs. 2a and 3). After dissociation in SDS sample buffer and running on SDS–PAGE, this high molecular weight peak was found to contain p101-ABRA and other lower molecular weight products reacting with mAB 3D5 (Fig. 2B, lane 3), suggesting that, in addition to producing proteolytic products of itself, p101-ABRA probably associates to form multimers with itself or other molecules. These aggregates were not dissociated by using 0.75 *M* NaCl in the Sephacryl buffer (Table II). Furthermore, only the lower molecular weight chymotryptic-like, p101-ABRA associated activity (peak I, ~90 kDa) entered nondenaturing (but reducing) PAGE gels (Fig. 5 and data not shown).

Likely candidates for molecules associating with p101-ABRA are erythrocyte spectrin and band 3. Spectrin is a large, sticky erythrocyte cytoskeletal protein that forms large complexes *in vitro* with a 37-kDa proteinase of *P. falciparum* (Deguercy *et al.* 1990). Band 3 is an erythrocyte transmembrane protein that appears to be a substrate for cleavage by a protease when erythrocytes are infected by *P. falciparum* (Crandall and Sherman 1991). Patarroyo's group has found that peptides containing the Lys–Glu–Lys (KEK) sequence bind to intact erythrocyte membranes (Calvo *et al.* 1991) and that antibodies against the KEK sequence may correlate with protective immunity (Molano *et al.* 1992). p101-ABRA contains eight KEK sequences (Weber *et al.* 1988), making it a good candidate for binding to an erythrocyte protein(s) which might be a substrate(s).

p101-ABRA was not detectable in uninfected erythrocytes (Fig. 2B, lane 2), and there was no chymotryptic-like activity of 90 to 110 kDa detected in uninfected erythrocytes (Figs. 2a, 3, and 5B). There was only a small amount of higher molecular weight chymotryptic-like activity detected in uninfected erythrocytes (Figs. 2a and 3), but attempts to separate it from the parasite chymotrypsin-like proteinase(s) of $M_r \geq 200$ kDa were not completely successful.

Chymostatin, an excellent inhibitor of chymotrypsin (Aoyagi and Umezawa 1975), at a concentration of 1 m*M* inhibited the activity of the proteinase by 88%; a lower concentration of 175 μ*M* inhibited the activity by 50% (Table III). Although this inhibitory chymostatin concentration (1 m*M*) is high compared to that used in

microbial systems, it is within the range used by other authors in investigations of the proteinases of *P. falciparum*. Schrevel *et al.* (1988) described a 68-kDa proteinase inhibited by a 1-m*M* concentration of chymostatin. The small but probably significant inhibitions by 1 m*M* leupeptin and antipain (Table III) suggest that p101-ABRA is not a classical chymotrypsin-like enzyme. Although p101-ABRA seems to cleave chymotrypsin substrates, those with Y or F in the carboxyl position, its lack of inhibition by PMSF further indicates that it is not a classical chymotrypsin-like enzyme.

Weber *et al.* (1988) found no sequence homology between p101-ABRA and other proteins in the data base, including chymotrypsin. However, after its signal peptide, p101-ABRA has 51 D, 8 H, and 27 S residues, some of which might form part of a catalytic triad as commonly found in serine esterases. Reinspection of the sequence has identified very limited homology with several proteases. For example, the sequence SGG, present in the active site of most serine esterases (Brenner 1988), is also present in p101-ABRA (residues 317–319), but with little surrounding homology. *P. falciparum* p113-SERA (SERP) is a serine-rich, soluble malaria antigenic protein located in the parasitophorous vacuole (Chulay *et al.* 1987). SERP is a putative protease, substrate unknown, based upon homology with several cysteine proteases such as papain, but with a serine instead of cysteine in the active site (Higgins *et al.* 1988; Eakin *et al.* 1988; Mottram *et al.* 1988). In the sequences at the bottom of the page the putative catalytic S or C are marked with an !, identical residues with a ¦, homologous residues with a :, and if adjacent with a \ or /.

Additional areas of homology between the SERP and papain-like cysteine enzymes involved a catalytically active H and N (rather than the D in classic serine proteases) separated by 12 to 24 residues; p101-ABRA has 15 pairs of H and N with similar spacing, but without much adjacent homologies (not shown). mAB 3D5 does not cross-react with SERP (Chulay *et al.* 1987).

To facilitate comparison with the putative chymotryptic-like proteolytic activity of p101-ABRA, the characteristics of a number of proteinases from *P. falciparum* parasites are given in Table IV. In *P. falciparum* proteinases are involved in at least two vital processes: processing of parasite antigens (Holder and Freeman 1982, 1984; Hall *et al.* 1984; Lyon *et al.* 1986; Delplace *et al.* 1988) and digestion of hemoglobin (Gyang *et al.* 1982; Vander Jagt *et al.* 1986, 1987; and Rosenthal *et al.* 1988). There is also evidence for the involvement of proteinases in the invasion of erythrocytes as suggested by studies using various proteinase inhibitors, including serum

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                                    !
chymotrypsin, rat     A S G V S S C M G D S G G P L V C Q K D G V W
                        ¦ : :   : : : ¦ ¦ ¦ \   :   \ : :
p101-ABRA             M D V V K N T L A Q S G G L G S N D L I N F L
                          \   :   :   : ¦   : : ¦ :  / ¦ :   :
SERP (P. falcip.)     Q V E D Q G N C D T S W I F A S K Y H L E T I
                        ¦ : : ¦ ¦ : ¦   : : ¦   ¦ : :   : : : ¦   ¦
papain (papaya)       P V K N Q G S C G S C W A F S A V V T I E G I
                                    !
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TABLE IV
Characterized Proteinases from *Plasmodium falciparum* Erythrocytic Stages

Source	Proteinase substrate	Inhibitors	Optimum pH	M_r (kDa)	Class	Reference
Schizonts	Suc-LLVY-, SY-, and MeOSuc-KLF-AFC	Chymostatin (CS)	7.0	101	Chymotryptic-like?	Nwagwu et al. (1992)
Schizonts and others	Erythrocyte ghosts, spectrin, band 4.1, and albumin	CS, LP, PMSF, E64, Hg^{2+}, Zn^{2+}, aprotinin	5.0	37	Serine or cysteine?	Deguercy *et al.* (1990), and Shrevel *et al.* (1990)
Schizonts and culture sup	Gelatin-PAGE[a]	NEM	(Neutral)	95[b]	Cysteine?	Schrevel *et al.* (1988)
Schizonts and merozoites	Gluconoyl-VLGK/R-AEC, gelatin-PAGE	CS, LP, AP, Hg^{2+}, Zn^{2+}, NEM, PHMS	7.4	68[b]	Cysteine	Schrevel *et al.* (1988), and Grellier *et al.* (1989)
Schizonts and merozoites	Gelatin-PAGE	CS, LP, PMSF	7.2?	76[c]	Serine?	Braun-Breton *et al.* (1988) and Braun-Breton and Pereira Da Silva (1988)
Trophozoites	Hemoglobin, Azocoll, gelatin-PAGE, ZVLR-, andZFR-AMC	LP, E-64, NEM, Z-FA-CH_2F	6.0	28	Cysteine	Rosenthal *et al.* (1987, 1988, and 1989)
Schizonts	Gelatin-PAGE	E-64, NEM	7.0?	35–40	Cysteine	Rosenthal *et al.* (1987)
Merozoites	Gelatin-PAGE	DIFP, PMSF	7.0?	75	Serine	Rosenthal *et al.* (1987)
Trophozoites	Denatured hemoglobin, albumin	Pepstatin, Hg^{2+}, phosphoramidon	4.5	5–10	Acid	Vander Jagt *et al.* (1986 and 1987)
Asynchronous	Hemoglobin	Pepstatin	3.5	48	Acid	Gyang *et al.* (1982)
Asynchronous	Alanine-NA	LP, Hg^{2+}, Zn^{2+}	7.5	186	Cysteine?	Gyang *et al.* (1982)
Asynchronous	*N*-acetylalanine-NA	LP, Hg^{2+}, Zn^{2+}	8.0	370	Cysteine?	Gyang *et al.* (1982)

Note. ? Indicates some uncertainty because of limited data. ND, not determined; Sup, supernatant fluid; Suc, succinyl; MeO, *O*-methyl; V, valine; L, leucine; G, glycine; R, arginine; K, lysine; S, serine; F, phenylalanine; Y, tyrosine; Z, benzyloxycarbonyl; AMC, amino-4-methyl-coumarin; AFC, 7-amino-4-trifluoromethyl coumarin; NA, *p*-nitroanilide; AEC, 3-amino-9-ethyl carbazole; PHMS, *p*-hydrozymercuriphenylsulfonic acid; NEM, *N*-ethylmaleimide; CS, chymostatin; LP, leupeptin; AP, antipain; E-64, trans-epoxysuccinyl-L-leucyl-amido-(4-guanidino)butane; DIFP, diisopropylfluorophosphate; PMSF, phenylmethylsulfonylfluoride.

[a] Gelatin substrate sodium dodecylsulfate polyacrylamide gel electrophoresis technique (Heussen and Dowdle 1980).

[b] The 68-kDa proteinase appears to be a processed product of a 105-kDa proteinase (Lawton *et al.* 1991).

[c] The 76-kDa proteinase can be activated by exogenous or endogenous (merozoite) phosphatidylinositol-specific phospholipases and appears to be a processed product of a slightly higher molecular weight precursor, probably located in the rhoptries.

α-chymotrypsin inhibitor and chymostatin (Tharavanij *et al.* 1983; Dejkriengkraikhul and Wilairat 1983; Hadley *et al.* 1983; Dluwski *et al.* 1986; Lyon and Haynes 1986; Delplace *et al.* 1988). Dluzewski *et al.* (1986) further implicated a putative chymotryptic-like enzyme in invasion by showing that the inhibition of invasion by chymostatin was counteracted if the erythrocytes had been first treated with chymotrypsin.

Two other parasite proteinases are of unknown relation to the work on chymotryptic-like proteinases, but of great interest for their probable role(s) during the process of invasion. One is p76, whose activation by an endogenous phosphatidyl inositol phospholipase C is associated with merozoite maturation and probably also the release of rhoptry contents during invasion into erythrocytes (Braun-Breton and Pereira Da Silava 1988; Braun-Breton *et al.* 1988). Another is a 68-kDa neutral proteinase that is inhibited by modifiers of cysteine groups and has been isolated from schizonts and merozoites of *P. falciparum* (Schrevel *et al.* 1988; Grellier *et al.* 1989). Schrevel's group reported inhibition of invasion using proteinase inhibitors based on peptidyl substrates modified with a nonhydrolyzable pseudopeptide bond at their usual trypsin-like cleavage site following a lysine (Schrevel *et al.* 1990; Mayer *et al.* 1991).

The putative chymotryptic-like activity of p101-ABRA described here appears to be distinct from any of these characterized proteinases of *P. falciparum* (Table IV).

Among the best candidate antigens of *P. falciparum* for inclusion in a malaria vaccine are those synthesized by schizonts and stabilized at the merozoite surface when schizonts mature in the presence of either proteinase inhibitors (Lyon and Haynes 1986) or immune serum (Chulay *et al.* 1981; Lyon *et al.* 1986a; Chulay *et al.* 1987). One of these antigens is p101-ABRA. Based on this study, it seems likely that p101-ABRA is a schizont-derived, soluble, neutral proteinase with chymotryptic-like activity of the type implicated in malaria invasion into erythrocytes. This study provides additional reason for further investigating p101-ABRA as one component in the development of a multicomponent malaria vaccine.

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